THE RELATION BETWEEN MATERNAL VITAMIN D STATUS IN PREGNANCY AND INCIDENCE OF INFECTION IN INFANTS UP TO ONE YEAR OF AGE

BY

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Submitted to the graduate degree program in Dietetics and Nutrition

and the Graduate Faculty of the University of Kansas

in partial fulfillment of the requirements for the degree of

Master's of Science.

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Date defended: 11/14/2011

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THE RELATION BETWEEN MATERNAL VITAMIN D STATUS IN EARLY PREGNANCY AND INCIDENCE OF INFECTION IN INFANTS UP TO ONE YEAR OF AGE

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Date approved: 10/24/2011

ABSTRACT

Research findings have suggested vitamin D enhances innate immunity in humans. Vitamin D deficiency among pregnant women is common, especially among women of darker skin. Studies have positively correlated newborn cord 25-hydroxyvitamin D (25(OH)D) with maternal serum 25(OH)D concentration during pregnancy. To our knowledge, no studies have addressed the associations between maternal serum 25(OH)D levels during pregnancy and incidence of infection in infants.

The aim of this study was to assess the relationship between maternal vitamin D status during pregnancy and incidence of infections in infants during the first six and twelve months of life. The number and type of medically-diagnosed illnesses and infections of 220 infants were collected, and the associations between maternal 25(OH)D concentrations and incidence of illness, total infections, and specific infection types (respiratory; skin; eye, ear, nose and throat (EENT); and others) were examined. Significant negative correlations were found between maternal 25(OH)D concentrations and the incidence of illness ($p = 0.022$), infection($p=0.033$), EENT ($p=0.043$), and skin infections ($p=0.021$) during the first six months, but not during the first 12 months.

Ethnic differences in this relationship was also examined in infants of African-American (AA) mothers (n=69) and in those who were not African-American (n=151) because we were aware that AA mothers had a higher incidence of vitamin D deficiency (plasma 25(OH)D < 50 nmol/L; 84.1% vs. 37.1%) and lower mean plasma 25(OH)D (35.20 ± 22.74 vs. 63.30 ± 31.96 nmol/L). Infants of AA mothers were more

likely than other women to have at least one incidence of any illness (p=0.013) and skin infection (p=0.007) during the first six months of life.

In infants of non-AA mothers, significant negative correlations were found between maternal 25(OH)D concentrations and incidences of skin (p=0.025) and EENT (p=0.026) at six months in the first six months of life. The relative risk for being diagnosed with at least one EENT and one skin infection with increasing 25(OH) concentrations trended lower in the first twelve months, but did not reach statistical significance.

Results suggest low maternal vitamin D status during pregnancy increases the risk of infection, in particular, the risk of skin and EENT infections in young infants. Few AA women had normal vitamin D status (4.3%), compared to women of other ethnic groups (26.5%), which could be one reason why their infants had more overall illnesses, infections, and skin infections during the first six month of life. Clinical trials to improve maternal vitamin D status during pregnancy could determine if there is a causal relationship between maternal vitamin D status and infant illness from infection, but caution should be taken since high concentrations of maternal 25(OH)D may be a risk factor for infant asthma.

ACKNOWLEDGMENTS

This project would not have been possible without the guidance of my mentor Susan Carlson, PhD. Special thanks for her constant help in developing my critical thinking skills. Many thanks for Debra Sullivan, PhD and Linda Griffith, PhD for serving on my thesis committee and for their suggestions and encouragement. I appreciate the time and effort of Amanda Foiles, MS, Elizabeth Kerling, MS, RD, and Jocelynn Thodosoff, MS, RD of the department of Dietetics and Nutrition for their assistance at medical record coding and development of my thesis database. Also thanks to Marlies Ozias and Ka Ian Chan who analyzed maternal plasma 25(OH)D concentrations. Assistance in statistical analysis was offered by Jill Shaddy, MS of the department of Dietetics and Nutrition and Jo Wick, PhD, of the department of biostatistics; many thanks for their help. Thanks to my friend Saddam Kanaan for his constant motivation and for providing me with a sense of home away from home. Across the miles, I thank my family, in Canada and in Lebanon, for the many hours of listening to me with love and complete faith in my potential.

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Chapter 1

INTRODUCTION

Vitamin D is well-known for its role in bone metabolism (1). A new area of research arose after the discovery of vitamin D receptors in human peripheral mononuclear leukocytes and in lymphocytes B and T (2). Vitamin D modulates immune function by suppressing adaptive immunity and stimulating innate immunity (3, 4). The active form of vitamin D [1, 25$(OH)_2$ D], promotes innate immune function by inducing the expression of the human cathelicidin antimicrobial peptide (CAMP) gene (5). In activated macrophages, Toll-like receptors mediate up-regulation of the vitamin D receptor gene and the vitamin D-α1-hydroxylase gene, to increase 1,25(OH)2D (6). Evidence on the role of vitamin D in modulating innate immunity is the basis for research on the role of vitamin D in infection.

Vitamin D deficiency is a pandemic with an estimated 1 billion individuals believed to be vitamin D deficient or insufficient (7). US Data from NHANES 2001-2006 indicate vitamin D deficiency occurs in 42% of non-pregnant women of child-bearing age and in 33% of pregnant women (8). In a cohort of 299 pregnant women in Kansas City metropolitan area, 86% of African American women, 56% of Hispanic women, and 35% of Caucasian women were found to be vitamin D deficient (25(OH)D < 50 nmol/L) (9). Maternal serum 25(OH)D during pregnancy is a significant determinant of the newborn's vitamin D status at birth. The newborn's cord 25(OH)D correlates positively with maternal serum 25(OH)D levels during pregnancy; reported correlations range from 0.79 to 0.898

(10-14). Reported cord blood concentration ranges from two-thirds to equal to maternal concentration (3, 10-14). There is very limited evidence for an increase in infection during infancy related to lower intrauterine vitamin D status. Camargo et al. (15) report an inverse association between cord-blood 25(OH)D and risk of respiratory infection by 3 months of age in 922 infants studied in New Zealand. A small study (n=25) of infants admitted to the intensive care unit for acute lower respiratory tract infections found significantly lower mean serum 25(OH)D compared to age-matched healthy controls (n=15) (16). No studies were located addressing the associations maternal plasma 25(OH)D levels during pregnancy and incidence illness, including infection, in infants.

Statement of Purpose:

The primary purpose of this study was to compare vitamin D status in pregnancy with the incidence of infections in their infants during the first six and twelve months of life. The women were from the Kansas City metropolitan area and participated in a clinical trial with infant follow-up that included documentation of illness from periodic interviews and medical records. We compared infant infections overall and by body system, e.g. respiratory and skin, with maternal vitamin D status. We independently compared illness in African-American and women of other ethnic groups, because the former group had a much higher incidence of vitamin D deficiency. The relationship between illness and maternal vitamin D status was also explored in infants of African-American women and of all other women.

Research Questions:

Primary research question:

Is there an association between maternal plasma 25(OH) vitamin D levels during pregnancy and the number of total medically-diagnosed infections experienced by that woman's infant during the first six and twelve months of life?

Secondary research questions:

1) Are specific types of infant infections (respiratory; skin; eye, ear, nose and throat infections) related to maternal vitamin D status during pregnancy?
2) Do infants of African-American and non- African American women have a similar relationship between maternal vitamin D status and either total or specific types of infections?

Chapter 2

LITERATURE REVIEW

INTRODUCTION

Vitamin D is well-known for its role in bone metabolism (1). The discovery of vitamin D receptors in non-bone tissues led to a new area of research on other functions of vitamin D. These functions can be described based on their general regulation effect on hormone secretion, immune function, or cellular proliferation and differentiation. In this review, the prevalence and causes of vitamin D deficiency, as well as the role of vitamin D in immune function are discussed. Focus is made on the evidence for vitamin D deficiency in pregnancy and the relationship between maternal vitamin D status during pregnancy and infant vitamin D status and immune function.

ASSESSMENT OF VITAMIN D STATUS

Vitamin D status is determined based on plasma 25-hydroxyvitamin D3 concentration (25(OH)D) (17). There is a debate on the optimal 25(OH)D concentration for defining deficiency. Common values indicating vitamin D deficiency and insufficiency are plasma concentrations of 25(OH)D less than 50 nmol/L and between 50- 75 nmol/L respectively. A plasma 25(OH)D of 75 -250 nmol/L indicates sufficient vitamin D status (1, 18-20). The Institute of Medicine (IOM) uses more conservative concentrations and divides vitamin D status into four categories based on plasma 25(OH)D concentration: risk of deficiency (<30 nmol/L), risk of inadequacy (30–49 nmol/L), sufficiency (50–125 nmol/L), and possibly toxic (>125 nmol/L) (21). The less conservative values for vitamin D status are based on the assumption that optimal serum 25(OH)D is the level at which parathyroid hormone concentration does not rise (22) and fractional calcium absorption plateaus at a serum 25(OH)D concentration of 75nmol/L (23, 24). A serum 25(OH)D concentration greater than 375 nmol/L indicates vitamin D intoxication (17).

VITAMIN D DEFICIENCY

VITAMIN D DEFICIENCY IN THE UNITED STATES OF AMERICA

Unless otherwise indicated, vitamin D status is defined based the 25(OH)D concentration categories used prior to the release of the recent IOM report on calcium and vitamin D dietary reference intakes (21). These concentrations are used because only one published study on vitamin D status in the US used the new IOM levels (25). Vitamin D deficiency is a pandemic, with an estimated 1 billion individuals being vitamin D deficient or insufficient (7). In the US, vitamin D deficiency has a different prevalence among different age groups. Of 380 infants and toddlers from a Boston urban clinic, 12.1% were vitamin D deficient (26). On the other hand, the prevalence among children and adolescents in the US ranges from 30 to 52% (27-30). Older adults in the US are at high risk of vitamin D deficiency, with a prevalence ranging from 40 to 100% (7).

Vitamin D deficiency is more prevalent among African Americans (31). In southern states, 53 to76% of non-Hispanic blacks are vitamin D insufficient, depending on age and gender. Prevalence in non-Hispanic white is 8 to 33% (32). Based on recent IOM categories, non-Hispanic Black Americans had a significantly higher prevalence of Vitamin D deficiency (32%) as compared to 3% in non-Hispanic whites (25). Lower 25(OH)D levels in African Americans are attributed mainly to the reduction in vitamin D synthesis with greater skin pigmentation (34). Melanin acts as a sunscreen by competing with skin 7-dehyrocholesterol for ultraviolet B (UVB) radiation. The higher the melanin content in skin, the lower is the biosynthesis of previtamin D3 (33). Another contributing factor is lower dietary intake of vitamin D among African Americans (34). Estimates of daily intake of vitamin D from food and dietary supplements are significantly lower in African American (6.9 ± 0.3 μg/d) than in Whites (8.4 ± 0.4 μg/d) based on analysis of NHANES data from 1999–2000 (34).

VITAMIN D DEFICIENCY WORLDWIDE

Vitamin D deficiency is prevalent in other parts of the world. One explanation for the geographical difference of vitamin D deficiency is the effect of latitude on vitamin D biosynthesis. Less UVB photons penetrate the ozone layer from October to March at latitudes above 37 degrees north and below 37 degrees south (35). Mean 25(OH)D levels are lower in people living in these areas; e.g., Boston (42 ºN) , northern Spain (43.5 ºN), northern France (49ºN), and Edmonton (52 ºN), compared to mean 25(OH)D levels in areas of latitudes between 37 º south and 37º north (30, 36, 37). Vitamin D biosynthesis may occur during the entire year in areas between 37º south and 37º north, such as in Puerto Rico (18 ºN), Los Angeles (24 ºN), and Buenos Aires (34 ºS) (35). Individuals living in countries where culture imposes the covering of the body and face have increased risk of vitamin D deficiency (38, 39). Few studies have addressed the effect of clothing on vitamin D status in the Middle East. Although the studies had small sample sizes, results suggest vitamin D deficiency is more common among veiled Kuwaiti and Lebanese women as compared to women without veils (38, 40). The observation of serum levels lower than 5 ng/ml (12.4 nmol/L) in most veiled women (38) suggests a major contribution of the veil to severe hypovitaminosis D. Prevalence of vitamin D deficiency in African countries is variable due to differences in latitude, climate, dress, and food availability across the continent (41). Dark skin pigmentation is a common risk factor for vitamin D deficiency among Africans regardless of the country (39). Melanin acts as a sunscreen by competing with skin 7-dehyrocholesterol for UVB radiations. Therefore, the higher the melanin content in darker skin, the lower is the biosynthesis of previtamin D3 (33).

MATERNAL VITAMIN D STATUS DURING PREGNANCY

Suboptimal vitamin D status is common among pregnant women in the US. Data from NHANES 2001-2006 indicate vitamin D deficiency occurs in 42% of non-pregnant women of child-bearing age and in 33% of pregnant women (8). Using the recent IOM-determined vitamin D categories for the same NHANES 2001-2006 data (21), 7% of pregnant or lactating women would be considered deficient, compared to 12% of women who are not yet pregnant or lactating (25). Bodnar et al. (42) reported the prevalence of vitamin D deficiency (25(OH)D < 37.5 nmol/L) and insufficiency (25(OH)D 37.5–80 nmol/L) in 200 white and 200 black pregnant women residing in Pittsburg, Pennsylvania. Vitamin D deficiency and insufficiency at delivery occurred in 29.2% and 54.1% respectively in African

American women, compared to 5% and 42.1% in white women. The study did not report vitamin D status in the overall sample, regardless of race. Forty-one percent of pregnant women (n=494) in South Carolina were vitamin D deficient (25(OH) D < 50nmol/L), and an additional 41% were insufficient (25(OH)D 50-80 nmol/L) during early pregnancy (43). Hamilton et al. (44) reported a similar distribution of vitamin D status of pregnant women in South Carolina (n= 559) with 48% of women being deficient and 37% insufficient. In a cohort of 299 pregnant women in Kansas City metropolitan area, 86% of African American women, 56% of Hispanic women, and 35% of Caucasian women were found to be vitamin D deficient (25(OH)D < 50 nmol/L) (9).

THE RELATION BETWEEN MATERNAL VITAMIN D STATUS DURING PREGNANCY AND VITAMIN D STATUS OF THE NEWBORN

Maternal plasma 25(OH)D concentration during pregnancy is significantly correlated with cord blood plasma 25(OH)D (45), with cord blood concentration being from two-thirds to equal maternal concentrations (3, 10-13, 39). A positive correlation exists between maternal and infant plasma 25(OH)D concentrations within 72 hours after birth (45). The positive correlation may be explained by the ability of serum 25(OH) D to cross the placenta. In contrast, maternal serum 1, 25(OH)$_2$D does not cross the placenta and does not contribute to the newborn's serum 1, 25(OH)$_2$D levels (46). Breast-fed infants rely on 25(OH)D stored during gestation to meet their requirements during the first few months of life, because breast milk is a poor source of vitamin D (46). Maternal vitamin D deficiency is a significant risk factor for infant vitamin D deficiency (45, 47). Mothers of rachitic infants have a higher prevalence of vitamin D deficiency compared to mothers of nonrachitic infants (48, 49).

VITAMIN D AS MODULATOR OF THE IMMUNE SYSTEM

Vitamin D plays a role in modulating both adaptive and innate immune functions. These roles were investigated after the discovery of vitamin D receptors in human peripheral mononuclear leukocytes and in B and T lymphocytes (2). The adaptive immune response is specific to the type of antigen presented to lymphocytes by macrophages and dendritic cells. It involves the release of cytokines and antibodies by B and T lymphocytes. The active form of vitamin D (1, $25(OH)_2D$) suppresses adaptive immunity by different mechanisms. Vitamin D inhibits the maturation of dendritic cells, reducing their ability to present antigens to lymphocytes T4. At the same time, 1, $25(OH)_2D$ has opposite effects on CD4 cell differentiation. Vitamin D inhibits CD4 cell differentiation to Th1 and Th17 cells and stimulates CD4 cell differentiation to Th2 and Treg cells (50, 51). This shift in CD4 cell differentiation is postulated to be the basis for a potential role of vitamin D in preventing and treating autoimmune diseases. Administration of 1, $25(OH)_2D$ and its analogues in non-obese diabetic mice prevent systemic lupus erythematosus, inflammatory arthritis, inflammatory bowel disease, and autoimmune diabetes. Analogs of $1,25(OH)_2D_3$ also treat type 1 diabetes and allergic encephalomyelitis, an experimental model for multiple sclerosis in mice (52). Observational studies have associated vitamin D deficiency and low vitamin D intake in humans with increased risk of Crohn's disease, type I diabetes, rheumatoid arthritis, and multiple sclerosis (53-56).

Randomized controlled trials testing the hypothesis that vitamin D can prevent autoimmune diseases are needed to prove the causality of vitamin D deficiency. On the other hand, suppression of the adaptive immune response by 1, 25$(OH)_2$D could reduce the body's ability to fight infectious agents; however, there is currently not published evidence for such an effect from published observational or interventional studies.

Innate immunity is characterized by non-specific and quick responses against pathogens, as the primary protective barrier between the body and the external environment. Epithelial cells, monocytes, phagocytes, and macrophages have transmembrane pathogen recognition receptors, called Toll-like receptors (TLRs). Once activated, innate immunity cells release reactive oxygen and nitrogen intermediates and antimicrobial peptides, including cathelicidin. The active form of vitamin D promotes innate immune function by inducing the expression of the human cathelicidin antimicrobial peptide (CAMP) gene (5). Active vitamin D (1, 25$(OH)_2$D) increases in activated macrophages by TLRs-mediated up-regulation of the vitamin D receptor and the vitamin D-α1-hydroxylase genes (6). Evidence that vitamin D modulates innate immunity led to research focused on the role of vitamin D in infection.

VITAMIN D AND INFANT INFECTION

Camargo et al. (15) reported an inverse association between cord-blood 25(OH)D and risk of respiratory infection in the first 3 months of life. They reported increased incidence of wheezing at 15 months, 3 years and 5 years in the same cohort of 922 infants. Neonates with cord-blood 25(OH)D less than 50 nmol/L have a sixfold higher risk of respiratory syncytial viral infection in the first year of life compared to those with 25(OH)D concentrations greater than 75 nmol/L (57). Newborns admitted to the intensive care unit for acute lower respiratory tract infections have significantly lower mean serum 25(OH)D than healthy controls (16).

Maternal vitamin D status during pregnancy is postulated to influence immunity of her offspring but more studies focused on its effect on asthma than on infections per se. Asthma is a disease of the adaptive immune system. New cell-culture studies suggest that asthma is a syndrome manifested due to an interaction between both innate and acquired immune systems (58). Although evidence supports a protective role of maternal vitamin D intake against infant wheezing (59-61), associations between maternal vitamin D intake and infant asthma are inconsistent. Devreux et al (60) did not find a significant association between maternal vitamin D intake from the diet or through dietary supplements and asthma in children (n=1751) at 5 years (OR=0.99 per quintile of energy-adjusted vitamin D intake, p=0.98). Maternal vitamin D intake from food was related to risk of asthma in Finnish 5- year-old children susceptible for type 1 diabetes (n=1669) (62). Maternal serum 25(OH)D greater than 30 ng/ml was associated with a 5-fold risk of asthma in children at 9 years of age compared to children with maternal 25(OH)D levels less than 12 ng/ml (63). In the same study, children exposed to maternal 25(OH)D levels greater than 30 ng/mL during late pregnancy were more likely as well to be diagnosed with atopic eczema at 9 months of age (63). The severity of viral lower respiratory tract illnesses in infants during their first year of life was not associated with maternal vitamin D levels at time of illness (64). However, the relevance of a potential association is not clear. The relationship between maternal serum 25(OH)D levels during pregnancy and incidence of infection in infants has not been investigated to our knowledge.

Chapter 3

METHODS

Overview:

This study used data collected from the Kansas University DHA Outcomes Study (KUDOS). The primary goal of the KUDOS study was to determine whether prenatal docosahexanoic (DHA) supplementation could improve pregnancy outcomes (eg. gestational length, birth weight, development of preeclampsia, gestational diabetes, etc) and infant cognitive development. The study design was a randomized double-blind, placebo-controlled clinical trial (registered as NCT00266825 at www.clinicaltrials.gov). The purpose of the present subordinate study was to assess the relationship between vitamin D status in a cohort of pregnant women from the Kansas City metropolitan area, and the total number of infections in their infants during the first year of life. The study also addressed if a specific type of infant infection is affected by maternal vitamin D status. The study investigated if infants of women of African descent have the same or different relationship between maternal vitamin D status and infection, compared to infants of non African American descent.

Sample

Two hundred and twenty infants of mothers who participated in the KUDOS trial are the sample. The KUDOS study included 350 women who met the following inclusion criteria: aged 16-35, BMI less than 40, pregnant with a single fetus at 8^{th} to 20^{th} week of gestation, and provided informed written consent. Women who had any serious illness, high blood pressure, or diabetes were excluded from the study. Until the day of delivery, subjects consumed either a daily 600 mg DHA capsule or a placebo vegetable oil capsule. After delivery, infants and their caregiver visited the study center for follow-up at the following periods of the infant's age: at 6 weeks and 4,6,9,10,12, and 18 months of age. For this vitamin D study, infants were included if they were born full-term (≥ 37 weeks of gestation), had completed their first year of life, their maternal plasma 25(OH) vitamin D concentrations were known, and medical records for the first year of life were available. Infants with serious illnesses (eg. cyctic fibrosis, HIV) that could compromise their susceptibility to infection were excluded from the study.

Setting

Women in the KUDOS trial were recruited between April 2006 and November 2009. The recruitment process took place at clinics at the University of Kansas Medical Center (Kansas City, Kansas), St. Luke's Hospital (Kansas City, Missouri), and Truman Medical Center (Kansas City,Missouri) and by email contact to KUMC employees. Some women were recruited through word of mouth. All women delivered their babies in hospitals in Kansas City metropolitan area, with the last infant born in May 2009. Infants returned with their caregiver to the study center at the University of Kansas Medical Center for follow-up visits at specific internals during infancy: at 6 weeks and 4, 6, 9, 10, 12, and 18 months.

Ethics

The Human Subjects Committee at the University of Kansas Medical Center reviewed and approved the research protocols, amendments, informed consent, and procedures of the KUDOS trial. This subordinate study uses blood measurements of nutrients and other assessments approved under the study. Data from medical records were authorized for the KUDOS trial and approved for release from infants' legal caregivers (Appendix A). This study is covered under the KUDOS HSC approval (HSC #10186). All participants provided informed consent at enrollment.

Procedures and materials:

The study design is a retrospective Cohort study. Maternal blood samples were collected at time of pregnant women enrollment, separated into plasma and red blood cell samples, and stored at minus 80 degrees Celsius. Plasma 25(OH)D concentrations were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Immundiagnostik AG, Bensheim, Germany) by the Kansas Intellectual and Developmental Disabilities Research Center (K-IDDRC) laboratory, as part of a previous study (9). The procedure for 25(OH)D assay is described in Appendix B.

Maternal race, number of pack years of smoking, and infant feeding (breastfeeding, formula-feeding, or both) during the first four months were self-reported by the mothers. Maternal gestational age at enrollment was determined from medical records. Maternal red blood cell phospholipid DHA concentrations at delivery were analyzed by gas liquid chromatography, after extraction, phospholipid separation by thin layer chromatography, and transmethylation with BF_3-methanal. Maternal DHA concentrations were analyzed in this study to control for potential confounding effects of maternal DHA status during pregnancy on study outcomes. Docosanoids derived from DHA are proposed to be anti-inflammatory agents, and may therefore modulate infant's immunity (65). Incidence, duration, and type of infections were recorded from medical records and cross-referenced with caregiver reports solicited during all follow-up visits to the study center. When the infant reached 1 year of age, medical records were requested from every clinic or hospital that the infant had attended, and faxed to the study center. During follow-up visits to the study center, the caregiver was asked about any infant illness, medication use, hospital/clinic visits, and corresponding treatment since the previous follow-up visit. Appendix C shows the adverse event log used during caregiver interviews. Any serious adverse event reported by the care giver (defined as any incidence of illness, hospitalization, or death) resulted in an immediate request for the infant's medical record and a report to the HSC. Infections reported by the parents and not confirmed in the medical records were not included in this study. Data on adverse events extracted from medical records were coded by type of illness and body system affected. Coding was within eleven main categories, as indicated in Appendix D: body as a whole; cardiovascular; eyes, ears, nose, throat (EENT); endocrine; gastrointestinal; metabolic and nutrition; musculoskeletal; nervous; respiratory; skin; and urogenital. For this study, the KUDOS database was used to capture the number and the kind of infection (s) each infant had in the first 12 months of life. Illness denoted any medically- diagnosed condition reported in the infant's medical record, whether it was an infection or not. Infections were derived from four categories: EENT, respiratory, skin; and all others (Appendix E).

The KUDOS database included needed data of demographic characteristics of the mothers, maternal delivery 25(OH) D concentrations and the number and types of medically-diagnosed illnesses and infections.

Statistical analysis:

Measurements of continuous variables are reported as mean ± standard deviation at 95% Confidence Intervals; number of cases and percentage (%) from total were used to report measurements of categorical variables. Differences in maternal baseline characteristics between AA and all other mothers were analyzed using independent sample t-test for continuous variables and Pearson Chi-Square for categorical variables. Plasma 25(OH)D distribution between the two racial groups did not meet the assumption of equal variances; therefore, Welch-Satterthwaite t-test was used to analyze differences in mean 25(OH)D concentrations. Prevalence of vitamin D deficiency, insufficiency, and sufficiency among African-American women and all other women are reported as percentage of total group (%). Comparison of prevalence within each vitamin D category was analyzed using Pearson Chi square test.

The relationships between maternal baseline characteristics for the two groups of women were analyzed using Spearman’s correlation test for continuous not-normally distributed variables (maternal DHA levels, maternal 25(OH)D levels, and pack years of smoking). Univariate analysis of variance was used to assess differences in 25(OH)D levels across infant feeding groups. Illness and infection types were not normally distributed. Therefore, non-parametric Spearman correlation test was used to analyze the correlations between 25(OH)D and infection types. Relative Risk (RR) was calculated to assess the risk of infections at different vitamin D categories. Significant differences in RR were assessed by Pearson Chi square test. Statistical significance was considered for two-tailed P value<0.05 for descriptive analysis (baseline maternal characteristics and incidence of infant illnesses) and one-tailed P value<0.05 for correlation tests. SPPS version 18 was used for statistical analysis.

Chapter 4

RESULTS

Maternal baseline characteristics

Two hundred and twenty women participated in this study; 69 (31.4%) were African American (AA) women and 151 (68.6%) were not. That group included 135 Caucasians, 12 Hispanics, and 3 others. Baseline maternal characteristics are summarized in Table 1.

TABLE 1

Baseline maternal characteristics

Maternal characteristics	All study subjects (n=220)	AA* women (n=69)	Other women (n=151)	AA compared to others (P Value)
Gestational age at enrollment (weeks)	14.6 ± 3.49	15.23 ± 3.45	14.30 ± 3.49	0.059 [a]
Maternal DHA concentrations (weight % from red blood cell phospholipids)	6.18 ± 2.27	5.42 ± 1.66	6.52 ± 2.43[d]	<0.001[a]
Plasma 25(OH) vitamin D concentrations (nmol/L)	54.41 ±32.10	35.20 ± 22.74	63.20± 31.96	<0.001[b]
Vitamin D status, n (%)				<0.001[c]
Deficient	114 (51.8)	58 (84.10)	56 (37.1)	

Insufficient	63 (28.6)	8 (11.60)	55 (36.4)	
Sufficient	43 (19.5)	3 (4.30)	40 (26.5)	
Infant feeding during 1st 4 months of life, n (%)				<0.001[c]
Exclusive breastfeeding	53 (24.1)	25 (36.76)	28 (18.79)[e]	
Exclusive formula feeding	69 (31.4)	5 (7.35)	64 (42.95)	
Both	95 (43.2)	38 (55.88)	57 (38.26)	
Smoked before pregnancy, n (%)	95 (43.18)	30 (43.48)	65 (43.05)	0.952[a]

* AA : African American Women
[a] P value calculated from independent sample t-test
[b] P value calculated from Welch-Satterthwaite t-test
[c] P value calculated from Pearson Chi-squared test
[d]217 subjects analyzed; excluded 3 subjects (2 African Americans and 1 non-African America) with unknown DHA concentrations.
[e] 217 subjects analyzed; excluded 3 subjects (1 African American and 2 non-African Americans) with unknown infant feeding type

Women were enrolled in the study during their first or second trimester of gestation, with a mean gestational age of 14.6 ± 3.49 weeks. Mean maternal 25(OH)D concentration at enrollment was 54.41 ±32.10 nmol/L, with 51.8% of the subjects being vitamin D deficient, 28.6% insufficient, and 19.5% sufficient. Univariate analysis shows that women who exclusively breastfeed their infants had significantly lower plasma 25(OH) D than other women (p=0.001). Plasma 25(OH) D was significantly correlated with red blood cell phospholipid DHA concentrations (r=0.232; p=0.001), but not with pack years of smoking.

Incidence of infant illness and infection

Table 2 and 3 summarize the number and percentage of infants with at least one medically-diagnosed illness or infection, during the first six and twelve months of life, respectively. During the first six months of life, 83.6% of all infants had at least one medically-diagnosed illness. By the time all infants reached twelve months of age, 95% of them had at least one illness. Diagnosis with at least one respiratory infection was the most prevalent diagnosis, in approximately 42% of all infants at six months and 62% at twelve months.

TABLE 2

Infants with at least one incidence of a medically-diagnosed illness or infection during the first six months of life

Illness or infection category, n (%)	All infants (n=220)	Infants of AA* mothers (n=69)	Infants of other mothers (n=151)	P Value**
Any illness	184 (83.6)	64 (92.96)	120 (79.47)	0.013
Any Infection	144 (65.5)	54 (78.27)	90 (59.60)	0.007
EENT Infection	67 (30.5)	26 (37.68)	41 (27.15)	0.115
Respiratory Infection	94 (42.7)	29 (42.09)	64 (42.38)	0.887
Skin Infection	46 (20.9)	22 (31.88)	24 (15.89)	0.007
Other Infections	26 (11.8)	10 (14.49)	16 (10.60)	0.406

*AA: African American

**P value calculated from Pearson Chi-square test for comparison of illness or infection incidence between infants of AA mothers and other mothers

TABLE 3

Infants with at least one incidence of a medically-diagnosed illness or infection during the first twelve months of life

Illness or infection category, n (%)	All infants (n=220)	Infants of AA* mothers (n=69)	Infants of non-AA mothers (n=151)	P Value**
Any illness	208 (95.00)	66 (95.65)	142 (94.04)	0.756
Any Infection	192 (87.70)	60 (89.96)	132 (84.42)	0.827
EENT Infection	128 (58.4)	36 (52.17)	92 (60.93)	0.201
Respiratory Infection	137 (62.6)	43 (62.32)	94 (62.25)	0.961
Skin Infection	87 (39.7)	32 (46.38)	55 (36.42)	0.173
Other Infections	54 (24.7)	18 (26.09)	36 (23.84)	0.739

*AA: African American

**P value calculated from Pearson Chi-square test for comparison illness or infection incidence between infants of AA mothers and other mothers

Tables 4 and 5 summarize the mean number of medically-diagnosed illnesses and infections during the first six and twelve months of life respectively.

TABLE 4

Mean ± standard deviation of medically-diagnosed illness or infections during the first six months of life

Illness or infection category, n (%)	All infants (n=220)	Infants of AA* mothers (n=69)	Infants of other mothers (n=151)	P Value**

Any illness	2.55 ± 2.16	3.10 ± 1.97	2.30 ± 2.20	0.008
Any Infection	1.4 ± 1.49	1.68 ± 1.49	1.26 ± 1.47	0.056
EENT Infection	0.43 ± 0.90	0.61 ± 1.26	0.35 ± 0.65	0.113
Respiratory Infection	0.64 ± 0.91	0.61 ± 0.83	0.66 ± 0.96	0.710
Skin Infection	0.24 ± 0.51	0.39 ± 0.65	0.17 ± 0.41	0.011
Other Infections	0.13 ± 0.38	0.19 ± 0.50	0.11 ± 0.31	0.204

*AA: African American

**P value calculated from independent sample t-test for comparison between infants of AA mothers and other mothers

TABLE 5.

Mean ± standard deviation of medically-diagnosed illness or infections during the first twelve months of life

Illness or infection category, n (%)	All infants (n=220)	Infants of AA* mothers (n=69)	Infants of non-AA mothers (n=151)	P Value**
Any illness	4.99 ± 3.67	5.62 ± 3.89	4.69 ± 3.54	0.094
Any Infection	3.30 ± 2.73	3.46 ± 2.78	3.22 ± 2.71	0.546
EENT Infection	1.05 ± 1.24	1.07 ± 1.44	1.05 ± 1.15	0.896
Respiratory Infection	1.27 ± 1.40	1.26 ± 1.31	1.27 ± 1.44	0.950
Skin Infection	0.64 ± 0.95	0.77 ± 0.97	0.58 ± 0.94	0.181
Other Infections	0.34 ± 0.66	0.38 ± 0.73	0.32 ± 0.63	0.577

*AA: African American

**P value calculated from independent sample t-test for comparison between infants of AA mothers and other mothers

Correlations between maternal 25(OH)D concentrations and infant outcomes

During the first six months of life, significant negative correlations were found between maternal 25(OH)D concentrations and the following infant outcomes: incidence of illness(p= 0.022), infection(p=0.033), EENT (p=0.043), and skin infections (p=0.021). At twelve months, none of the correlations reached statistical significance (Table 6). A scatter plot of the number of infections diagnosed during the first six months versus maternal 25(OH)D is represented in figure 2. The fit-line of the scatter plot shows a decreasing trend for number of infections with increasing maternal 25(OH)D levels.

TABLE 6

Univariate analysis of associations between maternal plasma 25 (OH) D concentrations and number of medically-diagnosed infant illnesses and infections during Six and Twelve Months of Life [1]

Illness/infection type	Correlation *	P value
Any illness		
At 6 months	−0.136	0.022
At 12 months	−0.053	0.220
Any infection		
At 6 months	−0.125	0.033
At 12 months	−0.023	0.365
EENT infections		
At 6 months	−0.116	0.043
At 12 months	0.008	0.453

Respiratory infections		
At 6 months	0.010	0.444
At 12 months	0.009	0.450
Skin infections		
At 6 months	−0.137	0.021
At 12 months	−0.064	0.172
Other infections		
At 6 months	−0.097	0.076
At 12 months	−0.060	0.189

[1] n= 220
*Correlation was determined using Spearman rank correlation coefficient (1-tailed)

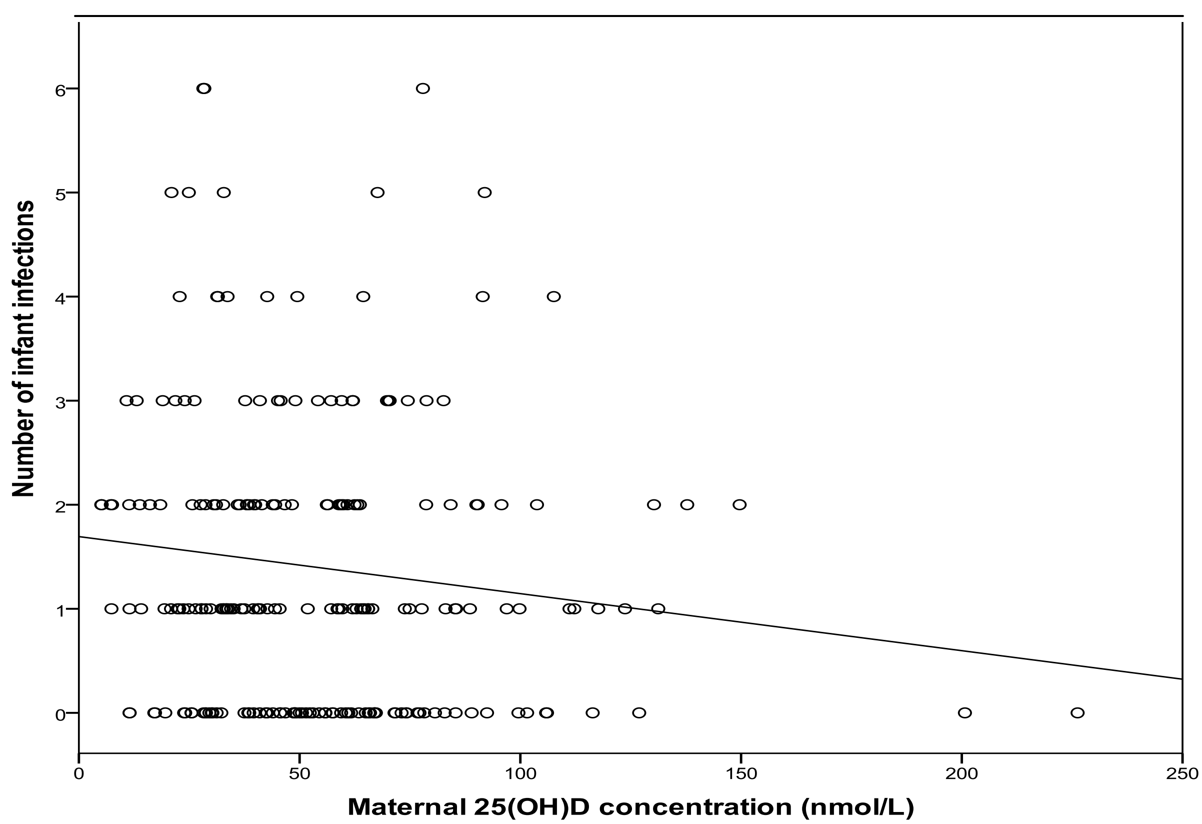

FIGURE 1. Scatter plot of the relation between maternal plasma 25(OH)D concentration and number of infant infections during the first six months of life

Relative risk for being medically-diagnosed with at least one illness or infection

Table 7 compares the risk of being diagnosed with at least one illness or infection at a particular maternal vitamin D status, to the risk of diagnosis at any other maternal vitamin D status. Differences in relative risk (RR) between vitamin D categories did not reach statistical significant at six and twelve months. However, there was a decreasing trend for RR for EENT and skin infections during the first six months of life. RR for EENT infections decreased from 1.22 in the deficient group (<50 nmol/L) to 0.64 in the sufficient group (>75 nmol/L) at six months. RR for skin infections decreased from 1.74 in the deficient group to 0.62 in the sufficient group at six months.

TABLE 7

Relative risk for medically-diagnosed infant illnesses and infections at six and twelve months of age according to maternal plasma 25(OH) vitamin D categories

	25(OH)D concentrations, nmol/L			
	<50	50-75	>75	P value*
Any illness				
At 6 months	1.04	0.96	1.00	0.776
At 12 months	1.00	0.98	1.04	0.635
Any infection				
At 6 months	1.16	0.86	0.95	0.284
At 12 months	1.00	0.94	1.08	0.392
EENT infections				

At 6 months	1.22	1.06	0.64	0.311
At 12 months	0.94	0.93	1.19	0.406
Respiratory infections				
At 6 months	0.97	1.00	1.04	0.972
At 12 months	0.96	1.10	0.96	0.725
Skin infections				
At 6 months	1.74	0.69	0.62	0.120
At 12 months	1.22	0.88	0.85	0.517
Other infections				
At 6 months	1.49	0.75	0.75	0.572
At 12 months	1.20	1.02	0.70	0.582

*P values were calculated from Pearson Chi Square test

Racial differences in maternal baseline characteristics

No significant difference in gestational age between AA women and other women was found at enrollment. AA women had significantly lower mean red blood cell phospholipid DHA concentrations and plasma 25(OH)D levels ($p<0.001$). AA women were more likely to breastfeed their infants exclusively during their first four months of life than others ($p<0.001$). The percentage of women who smoked before pregnancy was almost similar in both groups (Table 1).

Racial differences in maternal vitamin D status and its relationship with infant outcomes

Figure 2 shows the percent distribution of vitamin D status by race. The differences in percentages within each vitamin D category were statistically significant (p<0.001). AA women had a significantly higher prevalence of vitamin D deficiency, in 84.06% of AA women compared to 37.09% of other women. Only 4.35% of AA women in our study were vitamin D deficient, compared to 26.49% of other women.

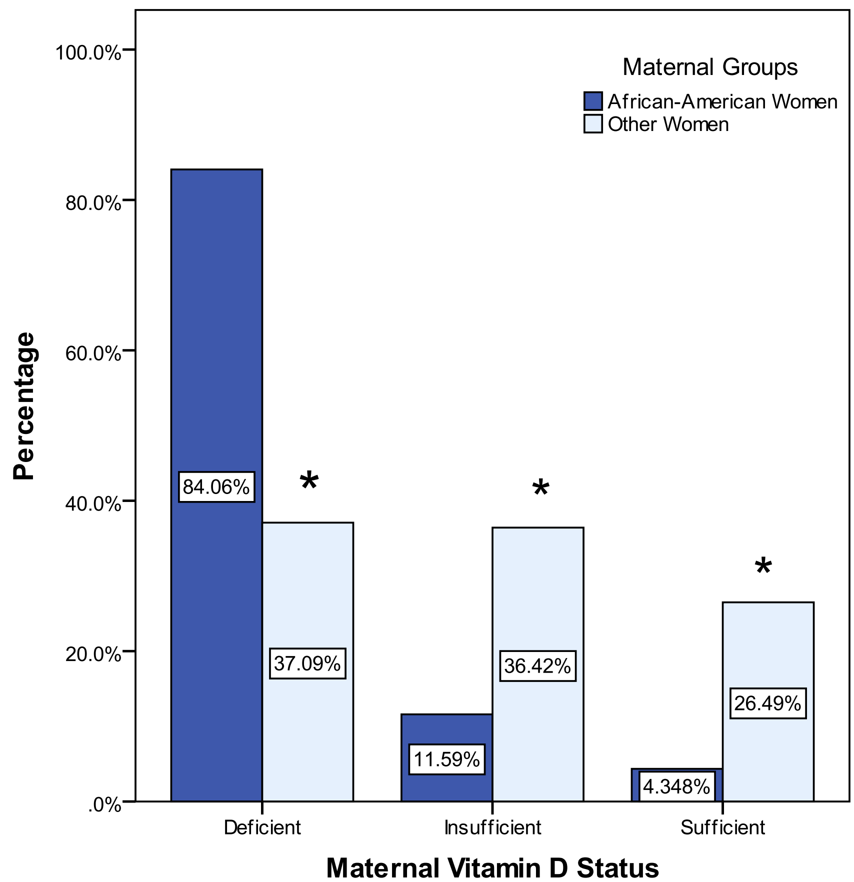

FIGURE 2. Maternal vitamin D status distribution (%) by race. * P<0.001

When comparing incidences of illness in infants by maternal race, we found that infants of AA mothers were more likely to have at least one incidence of any illness (p=0.013), any infection (p=0.007), and skin infection (p=0.007) during the first six months of life (Table 2). This difference was not statistically significant at twelve months (Table 3). Compared to infants of other mothers, infants of AA mothers had significantly higher number of illnesses (p=0.008) and skin infections (p=0.001) during the first six months of life (Table 4). At twelve months, there was no significant difference in mean number of illness and infections between the two groups (Table 5).

Correlations between maternal 25(OH)D concentration and infant illnesses within each racial group separately are shown in tables 7 and 8. Correlations in infants of AA mothers were not significant at six or twelve months of age (Table 7). At six months but not at 12 months, in infants of all other women, incidences of skin (p=0.025) and EENT (p=0.026) were significantly correlated with maternal 25(OH)D concentrations (Table 8).

TABLE 8

Univariate analysis of associations between maternal plasma 25 (OH)vitamin D and number of medically-diagnosed illnesses and infections at 6 and 12 months of age in infants of African American mothers [1]

Illness/infection type	Correlation *	P value
Any illness		
At 6 months	0.057	0.320
At 12 months	0.084	0.246
Any infection		
At 6 months	-0.002	0.493
At 12 months	0.038	0.379
EENT infections		
At 6 months	0.108	0.188
At 12 months	-0.039	0.374
Respiratory infections		
At 6 months	-0.082	0.253
At 12 months	-0.039	0.374
Skin infections		
At 6 months	0.119	0.166
At 12 months	0.161	0.093
Other infections		
At 6 months	-0.125	0.153
At 12 months	0.061	0.308

[1] n= 69

*Correlation was determined using Spearman rank correlation coefficient (1-tailed)

TABLE 9

Univariate analysis of associations between maternal plasma 25 (OH)vitamin D and number of medically-diagnosed illnesses and infections at 6 and 12 months of age in infants of non-African American mothers [1]

Illness/infection type	Correlation *	P value
Any illness		
At 6 months	-0.064	0.216
At 12 months	-0.028	0.369
Any infection		
At 6 months	-0.103	0.105
At 12 months	0.012	0.440
EENT infections		
At 6 months	-0.158	0.026
At 12 months	0.014	0.432
Respiratory infections		
At 6 months	0.001	0.497
At 12 months	0.028	0.366
Skin infections		
At 6 months	-0.159	0.025
At 12 months	-0.110	0.090
Other infections		
At 6 months	-0.093	0.127
At 12 months	-0.097	0.118

[1] n= 151

*Correlation was determined using Spearman rank correlation coefficient (1 tailed)

Chapter 5

DISCUSSION

Maternal vitamin D status during pregnancy

Suboptimal vitamin D status was common among pregnant women in our study. Mean maternal 25(OH)D concentration was 54.41 ±32.10 nmol/L, with 51.8% of the subjects being vitamin D deficient, 28.6%being insufficient, and 19.5% being sufficient. This distribution aligns with results from previous studies on vitamin D status in the pregnant American women (8, 9, 42-44, 66). The high prevalence of vitamin D deficiency may be attributed to reduced vitamin D biosynthesis caused by the latitude of Kansas City (39° 6' N) (35). Located above 37°N, less UVB penetrates the ozone layer between October and March, causing a significant decrease in vitamin D biosynthesis (35). Low vitamin D intake might have had a minor effect on vitamin D deficiency, because the contribution of vitamin D intake to vitamin D status is minimal (34). The discrepancy in vitamin D status between women of different skin color has been previously reported in the literature (25, 31-34, 39, 41). National data on vitamin D from 2001-2006 show that white women were less likely to be “at risk of deficiency” than black women, after adjusting for age and season (25). As anticipated, we found that the highest prevalence of vitamin D deficiency in our study was among AA’s, with a percentage of 84.1% as compared to 37.1% in other women.

We found women who exclusively breastfed their infants during the first 4 months of life were all vitamin D deficient (<50 nmol/L). They had significantly lower plasma 25(OH) D than other women in the study (41.71±4.30 vs. 58.47

±2.45 nmol/L). While the risk of low vitamin D status early in pregnancy is clearly not caused by breastfeeding, it does raise concern for high risk of infant hypovitaminosis D, especially as milk vitamin D reflects maternal vitamin D status.. If infants are not supplemented with vitamin D or adequately exposed to sun light, vitamin D content of deficient mothers alone would not be enough to meet their vitamin D requirements (46, 68). We do not have sufficient data on vitamin D supplementation in our infants to assess the consistency and adequacy of supplementation during the breastfeeding period.

Relationship between maternal 25(OH) D concentration and infant outcomes

The first year of life is critical for the infant's immune system development (71). Prenatal events can affect immune responses at birth, and acquisition of immune competence increases from birth to two years of age, with increased antigen exposure (71). We postulated that low maternal plasma 25(OH)D would be a "prenatal event" potentially altering the infant's immune function during the first year of life. Respiratory infection was the most prevalent diagnosis in infants of our study with approximately 42% of infants at six months and 62% at twelve months having at least one respiratory infection. Respiratory distress is among the top ten causes for infant death in the United States (72); however, we found no studies with which to compare this prevalence.

We found a significant negative correlation between maternal 25(OH)D concentration and incidence of illness and infection, specifically EENT and skin infection, during the first six months of life. Our study is the first to examine the association between maternal 25(OH)D concentrations during pregnancy and incidence of infection in the offspring during infancy. Previous studies examined the effects of maternal vitamin D intake during pregnancy and offspring immune outcomes, mainly asthma (59, 60, 62). Maternal 25 (OH) concentrations above 75 nmol/L during pregnancy were associated with infant asthma and atopic eczema (63).

Relative risk for being medically-diagnosed with at least one illness or infection

We were unable to detect statistically significant differences in relative risk (RR) for being diagnosed with at least one illness or infection among vitamin D categories. This study was a sub-study from the KUDOS trial, and consequently our study was not powered. Lack of adequate power and small sample size of mothers in the vitamin D sufficient group may have contributed to the insignificant RR outcome. We found a decreasing trend for RR for EENT and skin infections during the first six months of life. The Center for Disease Control (CDC) considers 3 or more ear infections annually as one of the determinants of health status (67). Our finding of reduced RR (RR=0.64) for EENT infections in infants of vitamin D sufficient mothers suggest that a sufficient vitamin D level during pregnancy is potentially a good determinant for infant health, based on CDC criteria.

Racial differences in the relationship between maternal 25(OH)D and infant outcomes

We also examined the relationship between maternal 25(OH)D concentration and infant infections, in each racial group separately. Our results suggest that during the first six months of life, infants of non-AA mothers have higher incidences of skin and EENT infections if their mothers have lower plasma 25(OH)D concentrations during pregnancy. We did not find a similar correlation in infants of AA mothers, but the group was smaller and the vast majority had vitamin D insufficiency or deficiency (95.6%).It is possible a similar correlation exists in infants of AA women, and this would have been found if our sample included a larger number of AA women with a wider range of vitamin D levels. This concept is reflected in the scatter plot of the number of infant infections versus maternal 25(OH)D concentrations (Fig. 2). Although the fit-line shows a negative relationship between the two variables, statistical significance is not achieved because few values of high maternal 25(OH)D are plotted in the graph. We investigated the likelihood that the difference in infection rates between infants of different maternal races may be due to factors other than maternal vitamin D levels. We found no significant difference in mean number of infections between infants of AA and other mothers, who were vitamin D deficient (data not shown). This means that differences in infection rates are less likely to be caused by factors other than maternal vitamin D levels (e.g. socioeconomic and educational factors).

Limitations

In our study, we used maternal 25(OH)D concentrations measured from blood samples collected at enrollment. Mean gestational age at enrollment was

14.6 ± 3.49 weeks. One limitation is the potential difference in 25(OH)D concentrations at enrollment and at delivery. However, Marwaka et al (73) found that the prevalence of vitamin D deficiency was not different across the three different trimesters. Prevalence of vitamin D deficiency among 48 non-supplemented pregnant women was reduced from 60 % during the first trimester to 48% at the second trimester and reduced by only 1 % at the third trimester (74).

The majority of our AA mothers were vitamin D deficient. Lack of a big sample size of AA women in the vitamin D sufficient group reduced our ability to evaluate maternal 25(OH)D and infant outcomes in that race. This study was a retrospective Cohort study, analyzing data that has already been collected as part of the KUDOS trial. Therefore, one limitation is that infant incidences of illness and infection were not the primary outcome measures. In our study, we only used illnesses that have been medically-diagnosed and excluded adverse events only reported by the parents. This would increase the accuracy of our dependent variables; however parents might not have reported all the visited clinics and hospitals for their infant treatments.

A potential limitation that was ruled out was an influence of DHA supplementation on infant illness and infection. Long chain fatty acids, especially eicosapentanoic acid and DHA, modulate host resistance to infection, mainly by modulating eicosanoid and lipid peroxidation pathways (75, 76). We found a significant correlation between maternal RBC phospholipid's DHA and 25(OH)D concentrations. However, maternal red blood cell phospholipid DHA concentration at delivery did not modify the relationship between maternal vitamin D status and infant infection (data not shown).

Conclusion and future directions

This study shows maternal vitamin D status has the potential to modulate infant immunity, especially during the first six months of life. This was the first study to associate maternal 25(OH) vitamin D levels during pregnancy with infant incidence of illness and infections. We found that maternal 25(OH) vitamin D concentrations are negatively correlated with incidence of infant illness, skin, and EENT infections during the first six months of life. We were unable to detect a significant correlation in infants of AA mothers, due to disproportional distribution of vitamin D status in our sample. Studies with vitamin D supplementation to improve vitamin D status are needed to determine if improving vitamin D status can reduce risk of infections during infancy. Randomized controlled trials are needed to determine cut-off maternal 25(OH)D levels for minimum risk of infant infection development. Long-term translational research is needed to validate whether health care policies addressing the correction of maternal vitamin D deficiency during pregnancy would reduce infant mortality and illness.

Chapter 6

SUMMARY

The aim of this study was to assess the relation between maternal vitamin D status during pregnancy and incidence of infections in infants during the first six and twelve months of life. We collected the number and type of medically-diagnosed illnesses and infections of 220 infants from the KUDOS trial, and we examined the associations between maternal 25(OH)D concentrations and incidence of illness, total infections, and specific infection types (respiratory; skin; eye, ear, nose and throat, and others). We also examined this relation in infants of African American and non-African American mothers separately.

Mean maternal 25(OH)D concentration at enrollment was 54.41 ±32.10 nmol/L, with 51.8% of the subjects being vitamin D deficient (plasma 25(OH)D < 50 nmol/L), 28.6% being insufficient (25(OH)D 50-75 nmol/L), and 19.5% being sufficient (25(OH)D > nmol/L). Among African American (AA) mothers (n=69), 84.1% were vitamin D deficient, and 37.1% of other mothers (n=151) were vitamin D deficient. Mean 25(OH)D concentration was significantly lower in AA mothers (35.20 ± 22.74 nmol/L) as compared to other mothers (63.30 ± 31.96 nmol/L) ($p<0.001$). Infants of AA mothers were more likely to have at least one incidence of any illness ($p=0.013$), any infection ($p=0.007$), and skin infection ($p=0.007$) during the first six months of life. Significant negative correlations were found between maternal 25(OH)D concentrations and the incidence of illness($p= 0.022$), infection($p=0.033$), EENT ($p=0.043$), and skin infections ($p=0.021$) during the first six months of life. At twelve months, none of the correlations reached statistical significance. After analyzing these correlations within each racial group separately, correlations in infants of AA mothers were no longer significant at six or twelve months. In infants of other mothers, significant negative correlations were found between maternal 25(OH)D concentrations and incidences of skin ($p=0.025$) and EENT ($p=0.026$) at six months only. The difference in relative risk (RR) of being diagnosed with at least one illness or infection at a different maternal vitamin D statuses did not reach statistical significant at six and twelve months. However, there was a decreasing trend for RR for EENT and skin infections during the first six months of life. RR for EENT infections decreased from 1.22 in the deficient group (<50 nmol/L) to 0.64 in the sufficient group (>75 nmol/L). RR for skin infections decreased from 1.74 in the deficient group to 0.62 in the sufficient group at six months.

This study supported our hypothesis that a negative association exists between maternal 25(OH)D concentrations in pregnancy and incidence of infection in infants during the first six months of life. A similar relationship was not found at twelve months. The study suggested increased incidence and risk of total infections, skin and EENT infections in infants of vitamin D deficient mothers of non-AA descent. The majority of AA mothers being vitamin D deficient reduced the statistical power to detect significant correlations in infants of AA mothers. This study did not support our hypothesis that infants of AA descent would have a similar association of maternal vitamin D status with incidence of infection. Further studies are needed to investigate a causal relationship between low vitamin D status during pregnancy and risk of infections during infancy.

Randomized controlled trials of large sample size and a wide range of vitamin D status distribution in AA and other mothers are needed to validate our result.

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APPENDIX A

CONSCENT FORM FOR RELEASE OF MEDICAL RECORDS

THE UNIVERSITY OF KANSAS HOSPITAL
CONSENT FOR THE RELEASE OF CONFIDENTIAL INFORMATION

I, ____ born on __________________________, hereby authorize ____ to disclose to:

The University of Kansas Medical Center
3901 Rainbow Boulevard MS 4013
Kansas City, Kansas 66160-7200
Attention: Infant/Toddler Nutrition Research Clinic Phone: (913) 588-5743; **Fax: (913) 945-6621**

the following information:____

____

____

____

____

I understand that my medical records (including any alcohol or drug abuse information) may be protected by Federal Regulations. I also understand that I may revoke this consent at any time except to the extent that action has been taken in reliance on it (e.g., probation, parole, etc.) and that in any event this consent expires automatically as described below.

SPECIFICATION OF THE DATE, EVENT, OR CONDITION UPON WHICH THIS CONSENT EXPIRES (if left blank this consent expires in one year)

____

EXECUTED THIS ____________________ DAY OF ______________________________, 20________

______________________________ (Witness)

______________________________ (Signature of Patient)

______________________________ (Signature of patient, guardian, or authorized representative)

______________________________ (Nature of relationship)

PROHIBITION ON REDISCLOSURE: THIS INFORMATION HAS BEEN DISCLOSED TO YOU FROM RECORDS WHOSE CONFIDENTIALITY IS PROTECTED BY FEDERAL LAW. FEDERAL REGULATIONS (42O FR PART 2) PROHIBIT YOU FROM MAKING ANY FURTHER DISCLOSURE OF THIS INFORMATION EXCEPT WITH THE SPECIFIC WRITTEN CONSENT OF THE PERSON TO WHOM IT PERTAINS. A GENERAL AUTHORIZATION FOR THE RELEASE OF MEDICAL OR OTHER INFORMATION IF HELD BY ANOTHER PARTY IS NOT SUFFICIENT FOR THIS PURPOSE. FEDERAL REGULATIONS STATE THAT ANY PERSON WHO VIOLATES ANY PROVISION OF THIS LAW SHALL BE FINED NOT MORE THAN $500, IN THE CASE OF A FIRST OFFENSE, AND NOT MORE THAN $5,000, IN THE CASE OF EACH SUBSEQUENT OFFENSE.

Drug Abuse Office and Treatment Act of 1972 (21 USC 1175) Comprehensive Alcohol Abuse and Alcoholism Prevention, Treatment, and Rehabilitation Act of 1970 (42 USC 4582)

APPENDIX B

25(OH)D ASSAY

Plasma 25(OH)D assay is performed using an enzyme-linked immunosorbent assay (ELISA) kit (Immundiagnostik AG, Bensheim, Germany) (77).

Procedure:

1) Pipette 30 μL of standards, controls, and plasma samples and place each into a labeled 1.5 mL Eppendorf tube.
2) Add 300 μL of releasing reagent into each tube and vortex briefly.
3) Incubate the tubes for 1 hour at 37 °C in a water bath or heating block.
4) Add 600 μL of sample dilution buffer into each tube and vortex briefly.
5) Pipette 100 μL of the mixture from each tube into 2 designated wells of the 96-well microtiter plate.
6) Add 150 μL of anti-25(OH)D antibody solution into each well and cover the plate.
7) Incubate for 18-22 hours at 8-10 °C in the dark.
8) Aspirate and wash the wells 5 times with 250 μL of diluted wash buffer using an 8-channel pipette. Using paper towel, remove the residual wash buffer in the wells after the last wash.
9) Add 200 μL of peroxidase-conjugated antibody solution into each well and cover the plate.
10) Incubate for 1 hour at room temperature while shaking with a horizontal microtiter plate shaker.
11) Repeat step 8.

12) Add 200 µL of TMB substrate into each well.

13) Incubate for 10-15 minutes at room temperature in the dark.

14) Add 50 µL of ELISA stop solution into each well.

15) Read the plate using a microtiter plate reader at 450 nm.

APPENDIX C

ADVERSE EVENT LOG SHEET

Adverse Event Log Sheet

Subject ID ______________________ Subject DOB ____/____/______

AGE AT EVENT: __________ months

Adverse Event ______________ Start Date: ____/____/_____

Code ______________ Stop Date: ____/____/_____

Ongoing: ____/____/_____

Serious: Yes No

Action taken (circle none or list as applicable):

None

Medications

Other, specify____________________

Hospital/Clinic seen at ____________________

Reported at visit ________________________

Coded ☐ Entered ☐ Checked ☐

APPENDIX D

LISTING OF ADVERSE EVENT COLDS BY BODY SYSTEMS

Listing of AE Codes in the Format Library

Body System	Code	Event
BODY AS A WHOLE	BODY024	ABNORMAL UMBILICUS
	BODY028	ACCIDENT
	BODY014	ALLERGY
	BODY036	ANAPHYLACTIC SHOCK
	BODY025	ANAPHYLAXIS
	BODY010	ANEMIA
	BODY007	APPARENT LIFE-THREATENING EVENT (ALTE)
	BODY017	ASPHYXIA
	BODY029	ASYMETRICAL FAT FOLD
	BODY015	BENIGN MASS
	BODY030	BREAST ENLARGEMENT
	BODY032	CARRIER BIOTIN DEFICIENCY
	BODY002	DEHYDRATION
	BODY016	DEVELOPMENTAL DELAY
	BODY021	DIAGNOSTIC PROCEDURE
	BODY034	DRUG ALLERGY
	BODY004	EXCESSIVE CRYING
	BODY003	FAILURE TO THRIVE
	BODY022	FEVER
	BODY038	FEVER OF UNKNOWN ORIGIN
	BODY033	FOOD ALLERGY
	BODY012	FUSSINESS
	BODY005	INFECTION
	BODY035	INSECT STING ALLERGY
	BODY001	IRRITABILITY
	BODY023	MICROCEPHALY
	BODY018	PAIN
	BODY027	PECTUS EXCAVATION
	BODY026	PLAGIOCEPHALY
	BODY019	REACTION TO VACCINE
	BODY008	RULE OUT SEPSIS
	BODY009	SEPSIS
	BODY020	SHORT STATURE
	BODY039	SPHEROCYTOSIS
	BODY006	SUDDEN INFANT DEATH SYNDROME (SIDS)
	BODY013	SURGERY
	BODY037	SYSTEMIC FUNGAL INFECTION
	BODY031	TEETHING
	BODY011	WEAKNESS

Listing of AE Codes in the Format Library

Body System	Code	Event
CARDIOVASCULAR	CARD007	ARRHYTHMIA
	CARD001	BRADYCARDIA
	CARD010	CARDIAC DEFECT
	CARD006	CARDIOMYOPATHY
	CARD002	CONGENITAL HEART DISEASE
	CARD003	COR PULMONALE
	CARD004	HEART MURMUR
	CARD005	HYPERTENSION
	CARD008	PERICARDIAL EFFUSION
	CARD009	TACHYCARDIA
ENDOCRINE	END003	ABNORMAL LABORATORY RESULT
	END004	ADRENAL HYPERPLASIA
	END001	ADRENAL INSUFFICIENCY
	END002	HYPOTHYROIDISM
METABOLIC AND NUTRITION	MAN009	ABNORMAL LABORATORY RESULT
	MAN005	ELECTROLYTE INBALANCE
	MAN008	FAILURE TO THRIVE
	MAN003	FETAL MALNUTRITION
	MAN011	FORMULA REJECTION
	MAN010	GLUTARIC ACIDEMIA TYPE 1
	MAN002	LACK OF APPETITE
	MAN006	MALNUTRITION
	MAN004	OSTEOPENIA/RICKETS
	MAN007	POOR WEIGHT GAIN
	MAN001	WEIGHT LOSS
MUSCULOSKELETAL	MS002	CRANIOSYNOSTOSIS
	MS004	DEFORMITY
	MS001	FRACTURE
	MS006	HIP CLICK
	MS007	HIP TIGHTNESS
	MS008	KNEE CLICK
	MS005	TORTICOLLIS
	MS003	TRAUMA

Listing of AE Codes in the Format Library

Body System	Code	Event
SKIN	SK014	ANGIOEDEMA
	SK021	ATOPIC DERMATITIS
	SK027	BACTERIAL SKIN INFECTION
	SK012	CHICKEN POX
	SK030	CONTACT DERMATITIS
	SK001	DIAPER RASH
	SK003	DRY SKIN
	SK004	ECZEMA
	SK015	ECZEMA/SEBORRHEA
	SK018	EDEMA
	SK023	ERYTHEMA
	SK028	FUNGAL SKIN INFECTION
	SK017	HEMANGIOMA
	SK006	IMPETIGO
	SK024	INCLUSION CYST
	SK022	INFECTION
	SK031	INSECT BITE
	SK026	INTERTRIGO
	SK019	IV INFILTRATE
	SK008	JAUNDICE
	SK011	NEONATAL ACNE
	SK025	NEVUS
	SK010	OTHER RASH
	SK013	PRURITIS
	SK005	SEBORRHEA
	SK007	STAPH INFECTION
	SK016	TRAUMA
	SK009	URTICARIA
	SK029	VIRAL SKIN INFECTION
	SK020	WART
	SK002	YEAST INFECTION

UROGENITAL	UG012	ABNORMAL GENITALIA
	UG016	ABNORMAL URINE
	UG014	CIRCUMCISION
	UG003	FETAL MALNUTRITION
	UG007	HYPOSPADIAS
	UG005	INGUINAL HERNIA
	UG009	LABIAL ADHESIONS
	UG002	LACK OF APPETITE
	UG013	PENILE ADHESION
	UG010	PENILE LESION
	UG011	RENAL CALCULUS
	UG008	UNDESCENDED TESTES
	UG004	URINARY TRACT INFECTION
	UG001	VAGINAL DISCHARGE
	UG006	VESICO-URETERAL REFLUX
	UG015	VULVITIS/VAGINITIS

RESPIRATORY	RESP025	ABNORMAL X-RAY FINDING
	RESP013	APNEA
	RESP008	ASTHMA
	RESP009	BRONCHIOLITIS
	RESP005	BRONCHITIS
	RESP017	BRONCHOPULMONARY DYSPLASIA
	RESP002	COUGH
	RESP004	CROUP
	RESP022	CYANOSIS
	RESP018	LARYNGITIS
	RESP012	PHARYNGITIS
	RESP007	PNEUMONIA
	RESP014	POSITIVE PRESSURE VENTILATION
	RESP020	PULMONARY EDEMA
	RESP021	PULMONARY HYPERTENSION
	RESP019	PULMONARY INSUFFICIENCY
	RESP016	REACTIVE AIRWAY DISEASE
	RESP015	REINTUBATION
	RESP010	RESPIRATORY DISTRESS SYNDROME
	RESP011	RESPIRATORY SYNCYTIAL VIRUS (RSV)
	RESP003	STREP THROAT
	RESP023	TACHYPNEA
	RESP024	TONSILLITIS
	RESP001	UPPER RESPIRATORY INFECTION (URI)
	RESP006	WHEEZING

Listing of AE Codes in the Format Library

Body System	Code	Event
EYES, EARS, NOSE AND THROAT	EENT020	ABNORMAL REFRACTION
	EENT039	ALLERGIC CONJUNCTIVITIS
	EENT037	ALLERGIC RHINITIS
	EENT038	ALLERGIC RHINO-CONJUNCTIVITIS
	EENT041	ALLERGIC SINUSITIS
	EENT031	BACTERIAL EYE INFECTION
	EENT026	BLIND
	EENT022	CATARACTS
	EENT036	CHOANAL STENOSIS
	EENT006	CONJUNCTIVITIS
	EENT008	CORNEAL ABRASION
	EENT029	EAR DRAINAGE
	EENT024	EAR WAX EXCESSIVE
	EENT018	EYE MOVEMENT DISORDER
	EENT023	EYELID INFECTION
	EENT013	HEARING DEFICIT
	EENT032	HEMANGIOMA
	EENT040	INFECTIOUS CONJUNCTIVITIS
	EENT043	INFECTIOUS RHINITIS
	EENT015	INFECTIOUS RHINITIS/SINUSITIS
	EENT042	INFECTIOUS SINUSITIS
	EENT012	LARYNGEAL EDEMA
	EENT034	LARYNGOMALACIA
	EENT025	LASER SURGERY/CRYOTHERAPY
	EENT021	MYRINGOTOMY/TM TUBES
	EENT002	NASAL CONGESTION
	EENT003	NASAL/TEAR DUCT OBSTRUCTIONS
	EENT030	OTITIS EXTERNA
	EENT001	OTITIS MEDIA
	EENT004	PURULENT RHINITIS
	EENT027	RETINAL DETACHMENT/HEMORRHAGE
	EENT014	RETINOPATHY OF PREMATURITY
	EENT011	RHINORRHEA
	EENT028	SEPTAL DEVIATION
	EENT010	SNEEZING/ITCHING
	EENT007	STAPH INFECTION IN EYE
	EENT019	SWALLOWING DISORDER
	EENT009	THRUSH
	EENT017	TONSILLECTOMY/ADENOIDECTOMY
	EENT016	TRAUMA
	EENT033	TUGGING AT EAR
	EENT035	VARIX
	EENT005	WATERY EYE

Listing of AE Codes in the Format Library

Body System	Code	Event
GASTROINTESTINAL	GI028	NECROTIZING ENTEROCOLITIS
	GI033	PERIANAL FISTULA
	GI011	PERIRECTAL ABSCESS
	GI042	PERSISTENCE OF UMBILICAL CORD
	GI016	PYLORIC STENOSIS
	GI034	RECTAL STENOSIS
	GI012	SALMONELLA IN STOOL
	GI013	SPITTING UP
	GI029	STOMATITIS
	GI026	STRAINING
	GI019	UMBILICAL HERNIA

APPENDIX E

LISTING OF ADVERSE EVENT BY GROUPS

Listing of Adverse Events by Groups			
EENT Infections	Respiratory Infections	Skin Infections	Other Infections
- Bacterial eye infection - Infectious conjunctivitis - Eyelid Infection - Infectious rhinitis - Infectious sinusitis - Otitis externa - Otitis media - Purulent rhinitis - Staph infection in eye - Thrush	- Bronchiolitis - Bronchitis - Croup - Pneumonia - Respiratory syncytial virus - Strep throat - Tonsillitis - Upper respiratory infection -	- Atopic dermatitis - Bacterial skin infection - Diaper rash - Fungal skin infection - Impetigo - Intertrigo - Pruritis - Staph skin infection - Viral skin infection - Yeast infection	- Acute gastroenteritis - Colitis - Gastrointestinal infection - Infectious gastroenteritis - Necrotizing enterocolitis - Salmonella in stool - Stomatitis - Meningitis - Urinary tract infection - Vulvitis/vaginitis - Sepsis - Rule out sepsis - Systemic fungal infection - Fever

*Illness category included all adverse events in Appendix C excluding: accident and trauma